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## **Efficient in vivo priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naive prostate cancer patients**

Karbach, J ; Neumann, A ; Atmaca, A ; Wahle, C ; Brand, K ; von Boehmer, L ; Knuth, A ; Bender, A ; Ritter, G ; Old, L J ; Jäger, E

**Abstract:** PURPOSE: NY-ESO-1, one of the most immunogenic tumor antigens, is expressed in 15% to 25% of metastatic prostate cancers. The immunological and clinical effects of vaccination with recombinant NY-ESO-1 protein combined with CpG as adjuvant were evaluated. **EXPERIMENTAL DESIGN:** In a phase I clinical study, patients with advanced prostate cancer were vaccinated with recombinant NY-ESO-1 protein (100 g) mixed with CpG 7909 (2.5 mg) every 3 weeks intradermally for 4 doses. Objectives of the study were the safety of the vaccine and changes of specific humoral and cellular immunological responses to NY-ESO-1 in relation to detectable NY-ESO-1 expression in the individual tumor. **RESULTS:** All 12 baseline sero-negative patients developed high-titer NY-ESO-1 antibody responses. B-cell epitope mapping identified NY-ESO-1 p91-110 to be recognized most frequently by vaccine-induced antibodies. Two patients developed significant antibody titers against the adjuvant CpG. NY-ESO-1-specific CD4+ and/or CD8+ T-cell responses were induced in 9 patients (69%). Five of these 9 patients did not express NY-ESO-1 in the autologous tumor. Postvaccine CD8+ T-cell clones recognized and lysed HLA-matched tumor cell lines in an antigen-specific manner. **CONCLUSION:** Our data provide clear evidence for the capacity of NY-ESO-1 protein/CpG vaccine to induce integrated antigen-specific immune responses in vivo and to efficiently prime CD8+ T-cell responses in NY-ESO-1 antigen-negative patients. Our results may also support further clinical vaccination protocols with NY-ESO-1 protein not only focused on the treatment of existing cancer, but also to prevent further development of NY-ESO-1 positive cancers in vivo.

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# Clinical Cancer Research



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## **Efficient in-vivo priming by vaccination with recombinant NY-ESO-1 protein and CpG in antigen naïve prostate cancer patients.**

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Running title: Vaccination with NY-ESO-1 protein and CpG 7909

Key words: Immunotherapy, Prostate cancer, NY-ESO-1 protein, Adjuvant, Integrated immune response

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## **Translational Relevance**

Antigen-specific cancer vaccines are designed to induce durable tumor-reactive immune responses to effectively control cancer growth. This study presents clear evidence for the immunogenicity of the NY-ESO-1 protein combined with CpG 7909 to prime antigen-specific naïve B- and T-cells, and to induce NY-ESO-1 specific, tumor-reactive CD8<sup>+</sup> T-cells in patients with metastatic prostate cancers. NY-ESO-1-specific humoral (100%) and cellular (69%) immune responses were elicited in vaccinated patients independent of any detectable NY-ESO-1 expression in the autologous tumor. Based on the capacity of the vaccine to effectively prime specific immune responses in patients with advanced cancer, we further consider the use of this vaccine in adjuvant and preventive treatment settings.

## Abstract

**Purpose:** NY-ESO-1, one of the most immunogenic tumor antigens, is expressed in 15-25% of metastatic prostate cancers. The immunological and clinical effects of vaccination with recombinant NY-ESO-1 protein combined with CpG as adjuvant were evaluated.

**Experimental Design:** In a phase I clinical study, patients with advanced prostate cancer were vaccinated with recombinant NY-ESO-1 protein (100 µg) mixed with CpG 7909 (2,5 mg) every 3 weeks intradermally for 4 doses. Objectives of the study were the safety of the vaccine and changes of specific humoral and cellular immunological responses to NY-ESO-1 in relation to detectable NY-ESO-1 expression in the individual tumor.

**Results:** All 12 baseline sero-negative patients developed high-titer NY-ESO-1 antibody responses. B-cell epitope mapping identified NY-ESO-1 p91-110 to be recognized most frequently by vaccine-induced antibodies. Two patients developed significant antibody titers against the adjuvant CpG. NY-ESO-1-specific CD4+ and/or CD8+ T cell responses were induced in 9 patients (69%). Five of these 9 patients did not express NY-ESO-1 in the autologous tumor. Post-vaccine CD8+ T-cell clones recognized and lysed HLA-matched tumor cell lines in an antigen-specific manner.

**Conclusion:** Our data provide clear evidence for the capacity of NY-ESO-1 protein/CpG vaccine to induce integrated antigen-specific immune responses in vivo and to efficiently prime CD8+ T-cell responses in NY-ESO-1 antigen-negative patients. Our results may also support further clinical vaccination protocols with NY-ESO-1 protein not only focused on the treatment of existing cancer, but also to prevent further development of NY-ESO-1 positive cancers in vivo.

## Introduction

NY-ESO-1 is the most immunogenic cancer-testis tumor antigen known to date. It is widely expressed in cancers including breast, bladder, prostate, melanoma, NSCLC, sarcoma and ovarian cancers where expression ranges stage-dependent from 20 to 80% of tumors. (1-4). The antigen often elicits spontaneous humoral and cellular immune responses against multiple MHC class I and II restricted NY-ESO-1 peptides in a proportion of patients with NY-ESO-1 positive tumors (5-10). Several clinical vaccine trials with different NY-ESO-1 formulations have shown that these vaccines are generally well tolerated and that cellular and humoral immune responses can be elicited in cancer patients (11-13). Although the initial trials were designed to primarily assess the safety and immunogenicity of the vaccine, a clinical benefit with extended time-to-progression intervals and regression of single disease parameters was observed in some patients who had developed detectable immune responses to the vaccine (14-15). Because NY-ESO-1 is presented on the surface of tumor cells only in the context of MHC molecules, it is important that the antigen-specific vaccine induces tumor-reactive NY-ESO-1 specific CD4/CD8 T-cells. Previous studies with recombinant NY-ESO-1 protein have shown that humoral and cellular immune responses were more efficiently induced when the antigenic protein was combined with a potent adjuvant as compared with protein alone (16-18). Of these adjuvants available, synthetic CpG oligodeoxynucleotides that bind to TLR9 expressed in B-cells and plasmacytoid dendritic cells, are likely to elicit proinflammatory cytokines, stimulate Th1-type immune responses and directly activate human B-cell proliferation and dendritic cell maturation resulting in an enhanced antigen-specific T-cell response (19-25). There is evolving evidence that NY-ESO-1 expression in the tumor changes from negative to positive during the progression of disease (26). In ovarian cancers, patients with stage 1 and 2 disease showed 14 % expression of NY-ESO-1 while patients with stage 3 and 4 disease showed an increase to 46% (27). In prostate cancer, which is known to be a relatively slow progressing disease, expression ranges stage dependant from 5-30%

(28-29). Therefore immunotherapeutic interventions at an early stage of disease in an adjuvant setting may improve immunosurveillance and possibly alter the clinical development. Patients in this study were vaccinated regardless of detectable NY-ESO-1 tumor antigen expression. Any vaccine-induced antigen-specific immune response in antigen-negative patients would underline the potential of the vaccine not only to induce antibodies and CD4<sup>+</sup> T-cell responses but also efficiently prime naïve CD8<sup>+</sup> T-cells. In the present study, we have assessed the immune responses elicited by vaccination with recombinant NY-ESO-1 protein mixed with CpG 7909. We show that prostate cancer patients receiving this vaccine develop integrated antibody and CD4<sup>+</sup>/CD8<sup>+</sup> T-cell responses to NY-ESO-1. A significant proportion of them did not express NY-ESO-1 in the tumor. These findings support the potential of recombinant NY-ESO-1 protein vaccine when combined with CpG to induce protective antibody and T-cell mediated immunity.

## **Material and Methods**

### **Investigational Agents**

Full-length human recombinant NY-ESO-1 protein was expressed in *E. coli* and purified using multi-step chromatography. The protein was formulated in 4 M urea containing 50 mM glycine and was provided by the Ludwig Institute for Cancer Research Ltd. CpG 7909, a single stranded 24 base phosphorothioate oligodeoxynucleotide (ODN) with the base sequence 5'TCG TCG TTT TGT CGT TTT GTC GTT 3', was synthesized and provided by Coley Pharmaceutical Group, Wellesley, MA, USA. Synthetic NY-ESO-1 peptides p157-165 and p157-170 were provided by the Ludwig Institute for Cancer Research Ltd.

All syntheses, productions, formulations and packaging of the investigational agents were performed in accordance with applicable current Good Manufacturing Practices and met the applicability criteria for use in humans.

## Study Design

This study was an open-label fixed-dose phase I study of immunization with recombinant NY-ESO-1 protein combined with CpG 7909 as an adjuvant in patients with high-risk stage D1 or advanced prostate cancer. Patients were included after giving written informed consent and received i.d. injections of 100 µg NY-ESO-1 protein mixed together with 2,5 mg CpG as an adjuvant every 3 weeks for 4 doses according to study protocol LUD03.024 which was approved by the Ludwig Institute for Cancer Research as well as by the medical and ethical committees of Hessen/Germany. Expression of NY-ESO-1 or LAGE-1 in autologous tumor samples was assessed by RT-PCR and/or immunohistochemistry retrospectively. For DTH testing, HLA-A2 and/or HLA-DP4 positive patients received intradermal injections of synthetic HLA-A2 and/or HLA-DP4 restricted NY-ESO-1 peptides p157-165 and/or p157-170, respectively, at baseline and on weeks 7, 13, 22 and 28 at a dose of 10µg peptide. DTH was assessed 48 hrs after each peptide injection. Blood samples were collected at baseline, prior to the second, third and fourth injection and three weeks after the fourth injection for clinical hematology, biochemistry, PSA levels and immune response assessments. Patients who demonstrated stable disease, minor, partial or complete response on week 13 were allowed to continue vaccinations until disease progression. In patients with mixed response, single progressive lesions could be resected and vaccination could be continued.

## Serological responses

NY-ESO-1 specific antibodies (Abs) were measured in the serum by standard ELISA assays on the day of each vaccination and 3 weeks after the last vaccination as previously described (26). Briefly, sera were added to 96-well plates (Nunc Maxisorb) coated with 2µg/ml (50µl/well) antigen o/n at 4°C and blocked with 2 % BSA/PBS. After incubation, plates were washed with PBS and specific antibodies were detected with alkaline phosphatase-conjugated anti-human IgG (Sigma



A9544). Following addition of pNPP (p-Nitrophenyl-phosphate) substrate and 3N NaOH stopping solution, absorbance was measured at 405 nm using an Anthos Laptec Instruments fluorescence reader. Sera were assessed over a range of dilutions from 1:100 to 1:400000. Vaccine-induced Abs were mapped with a panel of overlapping 20mer peptides (25µg/ml) spanning the whole protein sequence by ELISA. Immunoglobulin subclasses G1 and G3 were determined by Western blot analysis as described (26) using secondary antibody mouse anti-human IgG1/IgG3 at a dilution of 1:1000 (Zymed). In addition, sera were measured for serological responses against the CpG ODN adjuvant in standard ELISA at a coating concentration of 25µg/ml CpG 7909.

### **NY-ESO-1 specific T cells**

For testing in enzyme-linked immunospot (ELISPOT) and cytotoxicity assays, purified CD8<sup>+</sup> and CD4<sup>+</sup> T-cells were presensitized with adenoviral NY-ESO-1 (Ad2/ESO) -infected or NY-ESO-1 peptide pulsed irradiated autologous peripheral blood mononuclear cells (PBMC) depleted of CD4 and CD8 T-cells as described (30). Ad2/ESO was prepared by Dr. S.Yla-Herttuala (A.I. Virtanen Institute, University of Kuopio, Finland) for the Cancer Vaccine Collaborative. Presensitized CD4<sup>+</sup> and CD8<sup>+</sup> effector cells were tested on day 10-14 in  $\gamma$ -IFN ELISPOT assays for specific NY-ESO-1 reactivity using peptide-pulsed autologous antigen-presenting cells (EBV-transformed B-cells and dendritic cells) as target cells. A positive response was considered if the number of spots in the peptide-exposed well was two-fold or more higher than the number of spots in the unstimulated well, and if there was a minimum of ten (after subtraction of background spots) peptide-specific spots/25.000 T-cells or less if T-cell clones were used. For functional T-cell testing, cytotoxicity against NY-ESO-1 peptide-pulsed T2 cells and NY-ESO-1 expressing tumor cell lines was determined in <sup>51</sup>chromium release assays as described (31). In addition, tetramer analyses were performed in some patients using HLA PE-conjugated multimeric HLA-A2 and -Cw3 peptide complexes containing

NY-ESO-1 p157-165 and p96-104 synthesized at the Ludwig Institute of Cancer Research, Epalinges, Switzerland and used as described (15, 30). HLA-A2/HIVpol p476-484 and HLA-Cw3/ESOp92-100 tetramers were used as negative controls.

## **Results**

### **Patients**

Fifteen patients were enrolled in this trial, 3 of them were treated at Universitätsspital Zürich, Switzerland, and 12 patients were treated at Krankenhaus Nordwest, Frankfurt, Germany. Eleven patients had metastatic disease, four patients had high-risk stage D1 prostate cancer. All patients had advanced disease and thirteen patients had received previous hormonal and/or chemotherapeutic treatment. Two patients had NY-ESO-1 expressing tumors, eight were NY-ESO-1 negative, and in five patients NY-ESO-1 expression was not assessed. One patient (F-10) had NY-ESO-1 specific antibodies detectable at baseline. This patient had advanced disease including visceral and bone metastases. Thirteen patients completed at least 4 vaccinations and were therefore considered evaluable for immunological and clinical response. Two patients withdrew early from the study due to study drug related toxicity (ZH-3) and patients decision (F-2). Patient F-3 and F-6 continued vaccinations after 2 cycles since clinical benefit was observed and received the vaccine on the basis of compassionate single-patient protocols (SPP) for 15 and 20 additional injections according to the protocol, respectively. All patients received concomitant hormone ablative treatment. Summary of patient characteristics and clinical response are presented in Table 1.

### **Toxicity**

All patients were evaluable for toxicity. WHO grade 4 toxicities were not observed, grade 3 toxicity (hypotension) occurred in one patient (ZH-3). All patients developed local erythema and pruritus at the site of vaccination lasting 5-7 days.

Patient F-8 developed a systemic hypersensitivity reaction (grade 2) with generalized rash. In patient ZH-3 hypotension (grade 3), swelling, blister formation and central necrosis developed at the site of vaccination (grade 2) after the first injection. Hypotension became symptomatic two days after the vaccination and was considered possibly related to the vaccination. Due to the episode of hypotension and the strong inflammatory response following vaccination, the patient was withdrawn from the study.

### **DTH reactions**

DTH testing was performed with 10 $\mu$ g of NY-ESO-1 p157-165 and NY-ESO-1 p157-170 peptide in HLA-A2+ and HLA-DP4+ patients, respectively. No DTH reactions to these peptides were observed. Patient F-10 was HLA-A2 and DP4 negative and was therefore not tested for DTH reactions.

### **Serological responses after vaccination with rNY-ESO-1 protein and CpG.**

Following injections of 100  $\mu$ g rNY-ESO-1 protein together with 2,5 mg CpG intradermally every three weeks for 4 doses, one patient (F-10) showed NY-ESO-1 antibodies at baseline that increased from 6.400 at week 1 to a maximum of 51.000 (reciprocal titer) during vaccination. Patient ZH-3, who withdrew early, was only tested at baseline and at week 4 of vaccination. He remained sero-negative after one single vaccination. All other patients were sero-negative at baseline and developed significant serological responses against NY-ESO-1 during the course of vaccination. Specific IgG responses to NY-ESO-1 became generally detectable after the second injection between week 4 and 7 and titers further increased without generally reaching a plateau at a specific time point. Anti NY-ESO-1 IgG titers were variable among patients and did not correlate with the number of vaccinations. In addition, we analyzed serological responses against the adjuvant CpG 7909. Patients F-3 and F-8 developed vaccine-induced antibody responses against CpG with titers up to 1:25000 and 1:6400, respectively. Patient ZH-2 reacted with the adjuvant only

at one time point at study week 22 (Figure 1a). Maximum NY-ESO-1 Ab titers had reached 1:200000 after the first vaccine cycle in 2 patients, the other patients mostly developed maximum high titer Abs during the second cycle of vaccination. In some patients, persistence of vaccine-induced Abs was analyzed in follow up sera and remained detectable without further vaccination for up to 2 years after the last vaccination (Table 2). Vaccine induced NY-ESO-1 antibodies were of subtype IgG1 and IgG3 in all patients with the exception of patient F-10 who had preexisting IgG1 antibodies and who did not develop IgG3 antibodies. The IgG subtypes were compared to those found in patients with spontaneous NY-ESO-1 antibodies and in patients who seroconverted during vaccination with recombinant vaccinia/fowlpox NY-ESO-1 constructs (rV/rF-NY-ESO-1) in our previous trials. In both groups the dominant subtype was IgG1 only (Figure 1b). Epitope mapping of vaccine-induced antibodies with a panel of overlapping linear NY-ESO-1 20mer peptides showed recognition of B-cell epitopes located in the N-terminal part of the protein between p11-110. Within these regions, linear synthetic peptides p11-30, p51-70 and p91-110 were recognized by all vaccine-induced antibodies and were the first epitopes to be recognized during antibody development. Among those, antibodies against p91-110 often showed the strongest reaction in ELISA. No reactivity was found against epitopes located between region p120-180 with the exception of the C-terminal epitope p161-180 recognized by the serum of patient F-10. This B-cell epitope was so far more frequently found as the target epitope in patients with spontaneous NY-ESO-1 antibodies. In contrast to spontaneous Ab responses or responses induced by vaccination with recombinant rV/rF- NY-ESO-1 or CHP-NY-ESO-1, sera of patients in this study also reacted with peptide p101-120 (Figure 1c). This epitope was shown to be serologically recognized in the present study for the first time. We did not observed B-cell reactivity with peptide p81-100 in both groups. This is interesting as it is within the hotspot mid domain of major known T-cell recognition on the NY-ESO-1 protein.

### **NY-ESO-1 specific CD4+ T cell responses**

Before vaccination none of the patients had detectable CD4+ T cell responses against NY-ESO-1. After vaccination CD4+ T cell responses against different NY-ESO-1 epitopes were induced in 9 patients. The majority of CD4+ T-cells recognized sequences located in the three immunodominant distinct regions of the protein, corresponding to peptides p81-100, p119-143 and p151-180. Minor reactivity was found against some other peptides. Vaccine-induced CD4 epitopes are presented in Figure 2 and CD4 ELISPOT data of all patients are shown in Supplemental Table S1.

### **NY-ESO-1 specific CD8+ T cell responses**

At baseline, none of the patients had detectable CD8+ T cell responses against NY-ESO-1. During vaccination, detectable CD8+ T cell responses were induced in 6 patients. The epitopes recognized by CD8+ T-cells included p81-100, p91-110, p119-143 and p157-165, consistent with previous findings in patients with spontaneous responses as well as in patients immunized with other full-length NY-ESO-1 vaccines. Vaccine-induced CD8 epitopes are presented in Figure 2 and CD8 ELISPOT data of all patients are shown in Supplementary Table S1.

Two non HLA-A2+ patients (F-7 and F-10) developed CD8+ T cell responses against NY-ESO-1 p91-110 at week 10 of immunization. Patient F-9, who was HLA-A2 positive, showed a CD8+ T-cell response against NY-ESO-1 p157-165 at week 10 of immunization that was detectable also ex vivo with a frequency of 120 / 10<sup>6</sup> CD8+ T cells. Most T-cell responses developed early during the first cycle of immunization (Table 2). In patient F-3 and F-6, CD4/CD8 T-cells became detectable later during the second cycle. To evaluate the functional activity of vaccine-induced NY-ESO-1 specific T cells, we established CD8+ T cell clones from bulk cultures of patients F-7 and F-9 by limiting dilution using peptide-pulsed APC for stimulation. NY-ESO-1 p157-165 specific T-cell clones of patient F-9 showed reactivity against the NY-ESO-1 expressing HLA-A2+ tumor cell lines SK-

MEL-37 and NW-MEL-1045, CD8 T-cell clones from patient F-7 recognized NY-ESO-1 p91-110, and reacted with the NY-ESO-1 expressing tumor cell line MZ-MEL-7 as assessed in <sup>51</sup>chromium release assay (Figure 3A and B). CD8 T-cell clone 21 of patient F-7 was further analyzed by using overlapping 9-mer peptides covering the region p91-110 of the NY-ESO-1 protein. The exact minimal NY-ESO-1 peptide p96-104 was identified as the target epitope recognized by this T-cell clone. NY-ESO-1 epitope and HLA specificity of the T-cell clone was confirmed by HLA-Cw3/p96-104 tetramer staining, while the control tetramer HLA-Cw3/p92-100 did not bind to the T-cell clone (Figure 3B right).

In order to determine the stability and longevity of vaccine-induced NY-ESO-1 specific CD8 and CD4 T-cell responses, PBMCs were obtained in some patients after completion of vaccination. NY-ESO-1 specific vaccine-induced CD4+ T-cells in patient F-3, F-9 and ZH-2 were still detectable after 24, 3 and 2 months, respectively. Vaccine-induced CD8+ T-cells of patient F-3 specific for NY-ESO-1 p81-100 were detected during vaccination outside the standard protocol and during 2 years following completion of vaccination.

### **Clinical tumor response**

Of fifteen patients, 13 completed 4 vaccinations and were evaluable for clinical and immunological response. Most patients had advanced disease already at primary diagnosis. Eleven of the evaluable patients had stage IV disease at study entry. No early or rapid tumor progression and no partial or complete responses were seen. After four vaccinations, eight patients showed stable disease (F-1, F-3, F-4, F-5, F-6, F-10, F-12, ZH-2), three had progressive disease (F-7, F-9, ZH-1), and two patients remained free of detectable disease (F-8 and F-11). Clinical responses were determined by radiological staging and course of PSA values. All patients had concomitant antihormonal treatment. Patients F-1 and F-5 started first line antihormonal therapy at study entry. Therefore, the clinical development in these two patients may also be related to this treatment. Time to progression was



considered from the beginning of therapy to the date of disease progression. The median time to progression was 4,75 months, ranging from 0 to 16 months in all patients. Three patients (F-3, F-6, ZH-2) had extended time-to-progression intervals  $\geq 8$  months during or following vaccination. Patient F-3 experienced disease stabilization. Known bone metastases remained unchanged while PSA values decreased from 31,5  $\mu\text{g/l}$  at baseline to 12,8  $\mu\text{g/l}$  at week 10 and 11,8  $\mu\text{g/l}$  at week 25. Vaccination was therefore continued in a compassionate setting. Time until progression of disease was 8 months. Since then, the patient had received alternating sequences of antihormonal- and cytotoxic therapy. This patient continues to show a favorable clinical development since study entry and is alive with disease for more than 15 years since initial diagnosis. Patient F-6 achieved radiologically confirmed disease stabilization underlined by declining PSA values from 20,9  $\mu\text{g/l}$  at study entry to 12,7  $\mu\text{g/l}$  at week 16. The patient received continued vaccination according to the protocol for a total of 11 months as a compassionate treatment until disease progression. Considering a median time to progression of 6,5 months in patients with advanced prostate cancer under conventional chemotherapy, the clinical courses of these patients are considered outstanding.

### **Immune response and clinical tumor response**

Time to progression in patients who developed specific CD4 and CD8 T cell responses against NY-ESO-1 peptides (F-3, F-4, F-7, F-9, F-10, F-12) was 15.8, 4, 0, 0, 4 and 4.4 months, respectively. There was no correlation between detectable immune response and clinical stage or development of disease. Patients F-2 and F-10 had NY-ESO-1 expressing tumors. Patient F-2 completed only two vaccinations and was therefore not evaluable for immunological and clinical response. Patient F-10 had NY-ESO-1 serum antibodies at baseline. The antibody titer increased during vaccination and NY-ESO-1 specific CD8 and CD4 T cells were detected by ELISPOT analysis after 4 vaccinations. Time to progression in this patient diagnosed with liver, bone and lymph node metastases was 4 months.

## Discussion

Among the broad range of NY-ESO-1 specific immunotherapies evaluated during the past years, recombinant NY-ESO-1 protein vaccines are expected to induce a broader spectrum of epitope-specific T-cell immune response as compared with single or short peptide-based vaccine formulations that are limited in their use by the respective HLA restriction. In addition, NY-ESO-1 antibody responses were induced in almost all patients receiving NY-ESO-1 protein vaccination (16, 32-34). However, the biological role of NY-ESO-1 antibody in the development of the disease is not yet understood. Spontaneous NY-ESO-1 antibodies were found more frequently in patients with advanced tumor stages, suggesting that the duration of antigen exposure and antigen load play a major role for the induction of humoral immune responses, in particular since the antigen is not expressed on the surface of tumor cells (26). Therefore vaccination with NY-ESO-1 protein along with the adjuvant activity of CpG may overcome the insufficient immunogenicity of early stage tumors for inducing antibody responses. As detailed analysis of spontaneous and vaccine induced immune responses have shown, the development of NY-ESO-1 antibody is often associated with detectable NY-ESO-1 specific T-cell responses (8). This study presents the immunological and clinical results after vaccination with recombinant NY-ESO-1 protein combined with CpG 7909 in patients with high-risk stage D1 or advanced prostate cancer. The primary objective of the study was to evaluate the safety of the vaccine. Secondarily, the study evaluated the specific immunological response to NY-ESO-1 protein regardless of NY-ESO-1 expression in the autologous tumor. The vaccine was safe and well tolerated. One patient experienced WHO grade 3 toxicity (hypotension) possibly related to the vaccine. No other grade 3 or 4 toxicities were observed. DTH responses in all patients were negative and can probably be explained by the low dose of 10 µg of peptide injected for DTH testing. In previous studies, DTH tests were performed with 30 or 100 µg of peptide and DTH reactions were observed even if the peptide was used alone, without further adjuvant (35). Except for one patient who received only one single



vaccination, all baseline sero-negative patients developed high-titer NY-ESO-1 specific IgG antibody responses still detectable in some patients at a 1/200,000 dilution. The frequency of antibody induction was similar to other NY-ESO-1 protein vaccination studies using either different delivery forms of recombinant protein such as *cholesterol-hydrophobized-pullulan* (CHP-NY-ESO-1) (33), or protein alone combined with different adjuvants e.g. ISCOMATRIX, that forms lipid/saponin-based cage-like structures (16) or CpG (34). Out of these adjuvants, CpG 7909 that potently stimulates B-lymphocytes was likely to be responsible for the high antibody titers found in our study. Maximum vaccine induced antibody titers differed among patients but were generally high. Vaccine-induced antibodies were of IgG1 and IgG3 subtype. In contrast, IgG1 was the dominant subtype in patients after vaccination with rV/rF-NY-ESO-1 and CHP-NY-ESO-1, or in patients with spontaneous humoral immunity (36). Both, IgG1 and IgG3 subclass antibodies usually represent the humoral response to protein antigens and interact with the activating Fcγ-receptor IIa (CD32a) expressed on monocytes, B-cells and monocyte-derived dendritic cells. The antigenic epitopes recognized by antibodies in NY-ESO-1 protein vaccinated patients were similar to those recognized in cancer patients with spontaneous or CHP-NY-ESO-1 and rV/rF-NY-ESO-1 induced humoral immunity with the additional recognition of epitope p101-120 that was identified here for the first time in the majority of patients. Antibody responses against epitope p101-120 may be related to differences in protein formulation, the cDNA cloning systems or to the CpG and require further investigations. With respect to the immunodominance and hierarchies of recognition among NY-ESO-1 epitopes, it is remarkable that within the same hotspot mid-domain (p81-110) of T-cell reactivity, there is an analogy between B-cell and T-cell recognition of NY-ESO-1 epitope p91-110 while no B-cell reactivity with p81-100 was observed. Therefore it would be important to analyze if the antibody itself can recognize tumor cells directly that present this epitope on the cell surface in complex with HLA, even though NY-ESO-1 is an intracellular tumor antigen.

Of note, two patients developed antibodies against the CpG oligodeoxynucleotides used as adjuvant. The development of an immune response against the adjuvant itself has been previously described, e.g. against keyhole limpet hemocyanine (KLH) and anti-CpG-DNA antibodies have been previously described in ODN-injected mice (37). However, this is the first time that anti-CpG antibodies have been described against short unmethylated CpG dinucleotide sequences in humans. This finding was confirmed by re-analysis of our previous study LUD02.007, where three out of eight patients vaccinated with NY-ESO-1 peptide combined with CpG 7909 also developed CpG-directed antibodies (supp. Figure1). The CpG-directed antibodies do not seem to have a neutralizing effect since both patients developed high antibody titers against NY-ESO-1 protein and, in addition, generated NY-ESO-1 specific T-cell responses in the presence of CpG-directed antibodies.

In the present study most patients who developed vaccine-induced antibodies showed integrated CD4+ (9/13) and CD8+ (6/13) T-cell responses simultaneously. The frequency of T-cell response was in accordance with the findings of Valmori et.al. (34) who reported CD4+ T-cell responses in 17 of 18 patients and CD8+ T-cell responses in 9 of 18 patients after vaccination with the same antigenic protein combined with CpG and Montanide. In both studies patients were treated regardless of demonstrable expression of NY-ESO-1 in the autologous tumor. Focusing on tumor-antigen negative patients only, CD8+ T-cell responses were induced in 4 of 7 patients in our study and in 6 of 8 patients in the study by Valmori et.al. Therefore, the impact of Montanide for the immunogenicity of NY-ESO-1 protein/CpG vaccine formulation seems to be less significant as demonstrated in several peptide-based vaccine studies in which Montanide as an adjuvant was found to enhance antigen specific T-cell responses, documented by ex vivo detectable T-cell activation (38). CD8 T-cell responses in most cases occurred concurrently with CD4, and both concomitantly with high titer NY-ESO-1 specific antibodies, suggesting that specific antibodies may play an important role in priming of NY-ESO-1 specific CD8+ T-cells by forming immune complexes with the vaccine

protein that allow a better delivery of the protein to antigen presenting cells (39). Vaccine-induced CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses in our study were predominantly directed against the known immunodominant NY-ESO-1 regions p81-110, p119-143 and p157-170 that have been described previously (14, 34, 40). In addition, we could demonstrate functional activity of vaccine induced CD8<sup>+</sup> T-cell clones, which were able to recognize naturally processed NY-ESO-1 epitopes presented by tumor cells. Patient F-9 even exhibited an ex vivo detectable T-cell response against NY-ESO-1 p157-165. Direct ex vivo antigen-specific T-cells are rarely detected, but were found in several previously published studies either with NY-ESO-1 p157-165 peptide or Melan-A ELAGIGILTV analog peptide when the vaccines were combined with Montanide and CpG (15, 41, 42). These studies indicate the important role of CpG as an adjuvant in peptide-based and recombinant protein vaccines. Furthermore, if coadministered with the NY-ESO-1 protein, CpG ODNs quite possibly initiate DC activation and thus may enhance the development of specific CD8<sup>+</sup> T-cell responses. Interestingly, 3 patients developed NY-ESO-1 specific IgG antibodies without detectable NY-ESO-1 specific CD4 T-cells and all CD4 responder developed antibody responses prior to the CD4 response. This suggests, that CpG may be able to bypass the antigen-specific T-helper cell response required for the subclass switch from IgM to IgG.

In summary, our study demonstrated that NY-ESO-1 protein vaccination at a dose of 100 µg combined with CpG ODNs is highly immunogenic and able to elicit primary antigen-specific humoral, CD4<sup>+</sup> and CD8<sup>+</sup> T- cell immune responses in patients with or without NY-ESO-1 expressing tumors. This vaccine approach may open the possibility for the design of further vaccinations in an adjuvant setting including both, target-antigen specific and antibody-based immunotherapies to protect patients with high-risk tumor stages from tumor expansion.

## Tables

### Table 1: Patient characteristics and clinical response

### Table 2: Summary of vaccine-induced immune responses

## Figure legends

### Figure 1: Serological responses to the vaccine

A: NY-ESO-1 antibody responses (blue line) were assessed in ELISA at baseline and at indicated study weeks during vaccination. OD values at 405nm at a serum dilution of 1:400 were shown. Anti-CpG antibody responses are shown by the green line. As a control sera were tested against the unrelated MAGE-3 protein (pink line).

B: Western blot analysis for the determination of antigen specific IgG subclass antibodies in patients after NY-ESO-1 protein vaccination. Data from patients with spontaneous NY-ESO-1 or rV/rF-NY-ESO-1 induced antibodies were shown in the lower line. Patient's sera were added at a dilution of 1:250.

C: Comparative analysis of B-cell epitopes recognized by NY-ESO-1 protein vaccine-induced antibodies (left graph) and by rV/rF-NY-ESO-1 vaccine-induced or spontaneous antibodies (right graph). Linear 20mer overlapping peptides spanning the NY-ESO-1 protein sequence were determined using patients sera at a serum dilution of 1:400 and displayed as a comparison of single epitopes with regard to the total sum of OD values recognized by all patients after subtraction of background values.

### Figure 2: NY-ESO-1 epitopes recognized by post-vaccine T-cells

CD4<sup>+</sup> and CD8<sup>+</sup> T-cells were prestimulated with Ad2/ESO infected or NY-ESO-1 peptide loaded PMBCs and tested in  $\gamma$ -IFN ELISPOT on peptide loaded autologous or allogeneic APC. Before vaccination, antigen-specific T-cells were not detected.

### **Figure 3: NY-ESO-1 protein vaccination elicits antigen-specific and tumor-reactive CD8+ T-cells**

A: Specific cytotoxicity of post-vaccine T-cell clone 8 from patient F-9 against HLA-A2/NY-ESO-1 expressing tumor cell lines SK-MEL-37 and NW-MEL-1045. Right: NY-ESO-1 p157-165 specificity of T-cell clone 8 was confirmed by flow cytometry using p157-165/HLA-A2 tetramer. As negative control, T-cells were stained with HLA-A2/HIV multimers (ILKEPVHGV).

B: Specific cytotoxicity of CD8 T-cell clone 21 from patient F-7. NY-ESO-1 p96 - 104 (FATPMEAEL) was recognized on peptide pulsed autologous EBV-B-cells. Cross-reactivity against naturally processed NY-ESO-1 on tumor cell line NW-MEL-9 is shown. NY-ESO-1 negative tumor cell line NW-MEL-12, autologous EBV-B-cells and K562 were not recognized. Right: Epitope specificity was confirmed by tetramer staining using Cw0304/p96-104 multimers.

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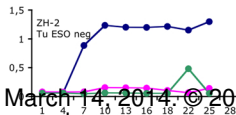
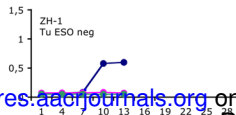
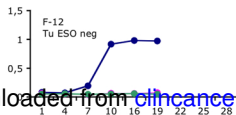
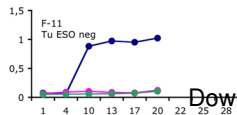
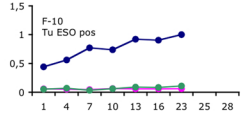
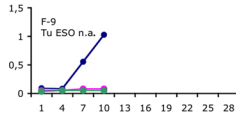
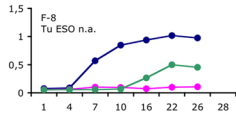
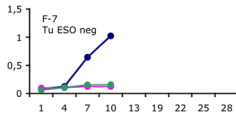
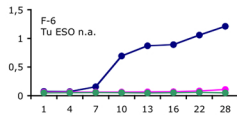
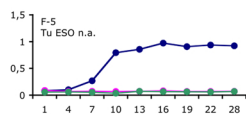
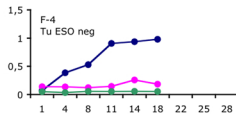
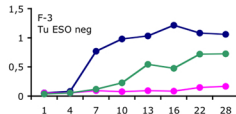
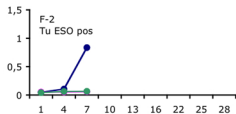
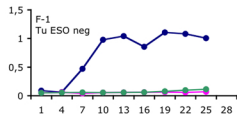
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IgG1

IgG3

F-3

F-4

F-5

F-6

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NW29

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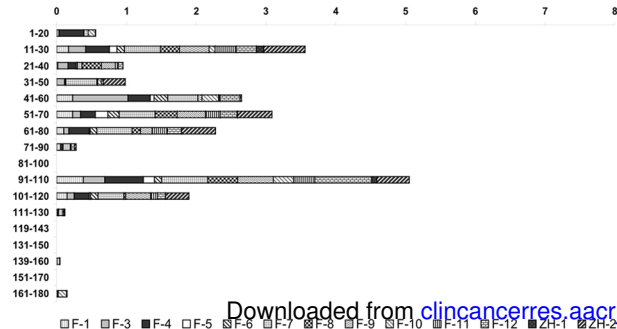
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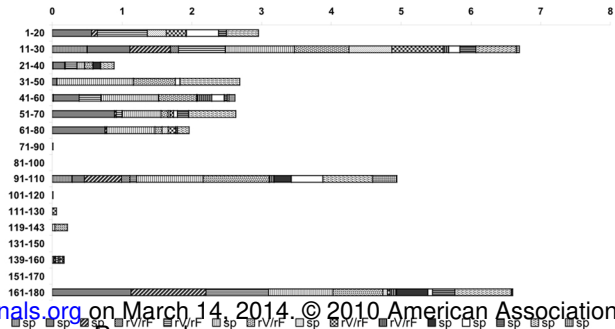
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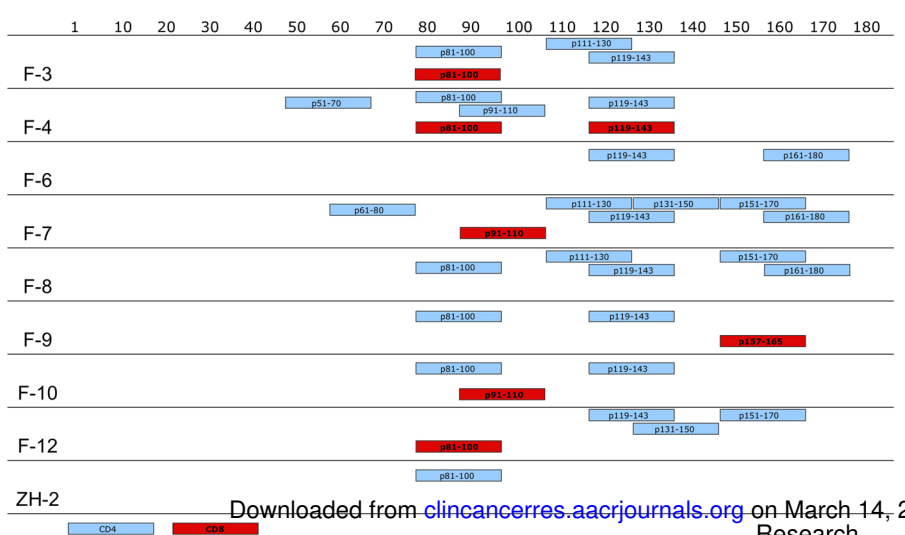
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 patients with  
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 patients  
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## NY-ESO-1 protein-vaccinated patients

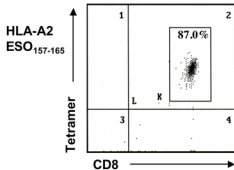
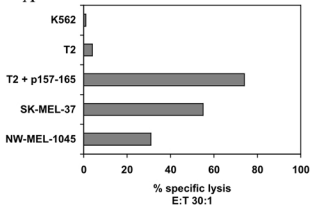


## rV/rF-NY-ESO-1 vaccinated patients and patients with spontaneous antibody

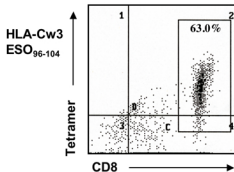
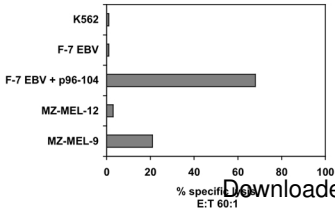




*A*



*B*



Patient	Initial stage at diagnosis	Stage at study entry	Metastases	Tumor NY-ESO-1 expression (IHC)	Biopsy site	No. of Vacc.	Best Response	Time to Progression	Overall Survival	Total Survival	Toxicity (grade) ¶
F-1	IV	IV	B, LN	-	Prostate	8	SD	5.9	8.3	10	L(2), S(1)
F-2	II	IV	NED	+	Bladder	2	n.e.	n.e.	10.8	168	L(2), S(2)
F-3	II	IV	B	-	Prostate	15	SD	12	64.3 +	180 +	L(2), S(1)
F-4	unknown	IV	B	-	LN	5	SD	4	34.3	96	L(2), S(1)
F-5	IV	IV	B, LN	n.a.		8	SD	6.2	6.3	9	L(2), S(1)
F-6	unknown	IV	B	n.a.		20	SD	12.4	63.4	72	L(2), S(1)
F-7	III	III	NED	-	Prostate	4	PD	2.5	14.5	108	L(2), S(2)
F-8	III-IV	IV	NED	n.a.		7	NED	5.1	33.8	96	L(2), S(2) §
F-9	III	IV	B	n.a.		4	PD	3	18.2	108	L(2), S(2)
F-10	unknown	IV	Li, LN, B	+	Prostate	6	SD	4	8	60	L(2), S(2)
F-11	III	III	NED	-	Prostate	6	NED	n.e.†	7.7	120	L(2), S(2)
F-12	IV	IV	LI, LN	-	Prostate	6	SD	4.4	11.1	12	L(2), S(2)
ZH-1	III	IV	LN	-	Prostate	4	PD	3	3.5	61	L(2), S(2)
ZH-2	IV	IV	B, LN	-	Prostate	8	SD	10.5	54	132	L(2), S(2)
ZH-3	III	IV	B	-	Prostate	1	n.e.	n.e.	25	36	L(2), S(3) §

¶ L: local injection reaction including reddening, swelling, pain

S: related systemic toxicity including fever, chills, flu like-syndrome, headache, myalgia, athralgia, sweating, tachycardia, nausea, diarrhoea

§: reaction leading to study termination: F-8 hypersensitivity CTC 2; ZH-3 hpotension CTC 3

†: patient started new treatment prior to progression

LI, liver; LN, lymph node; B, bone; n.e., not evaluable; n.a., not applicable NED, no evidene of disease; Vacc., vaccinations, all time dates in months.

Time to progression, Overall survival, Total survival: in months; IHC: Immunohistochemistry

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Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

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Patient	ESO Tumor	No. of Vaccines	HLA A2/DP4	DTH Peptide	maximum NY-ESO-1 antibody titer				CD4		CD8	
					pre	post cycle 1	post cycle 2	follow up sample	pre	post	pre	post
F-1	-	8	A2/DP4	-	-	25,600	51,200		-	-	-	-
F-2	+	2	A2/DP4	-	-	25,600			n.e.	n.e.	n.e.	n.e.
F-3	-	15	A2/DP4	-	-	51,200	200,000	25.600 (27 m)	-	+ (w28)	-	+ (w28)
F-4	-	5	DP4	-	-	51,200	51,200	6.400 (20 m)	-	+ (w12)	-	+ (w18)
F-5	n.a.	8	DP4	-	-	25,600	51,200		-	-	-	-
F-6	n.a.	20	DP4	-	-	25,600	200,000		-	+ (w28)	-	-
F-7	-	4	DP4	-	-	200,000		12.800 (11 m)	-	+ (w 7)	-	+ (w10)
F-8	n.a.	7	DP4	-	-	25,600	100,000	1.600 (18 m)	-	+ (w10)	-	-
F-9	n.a.	4	A2	-	-	200,000		800 (14 m)	-	+ (w 7)	-	+ (w10)
F-10	+	6	-/-	n.a.	6,400	25,600	51,200		-	+ (w10)	-	+ (w10)
F-11	-	6	A2/DP4	-	-	51,200	100,000		-	-	-	-
F-12	-	6	DP4	-	-	25,600	51,200	12.800 (12 m)	-	+ (w19)	-	+ (w19)
ZH-1	-	4	DP4	-	-	6,400			-	-	-	-
ZH-2	-	8	DP4	-	-	100,000	200,000	100.000 ( 5 m)	-	+ (w 7)	-	-
ZH-3	-	1	A2	-	-	-			n.e.	n.e.	n.e.	n.e.

DTH, delayed-type hypersensitivity to NY-ESO-1 p157-165/ DP4 peptide; pre, prevaccination; post, postvaccination; m, months; w, study week;  
 n.a., not applicable; n.e., not evaluable;

Values in brackets represent the study week T-cell responses were detectable and antibody persistence in months for follow up samples